APPENDIX A - SPECIFICATION/CLAIM AMENDMENTS INCLUDING NOTATIONS TO INDICATE CHANGES MADE
Applicant: Stice
Title: EFFECTIVE NUCLEAR REPROGRAMMING IN MAMMALS
Serial No.: 09/809,662
Docket No.: 235.0032 0101

Amendments to the following paragraphs are indicated by underlining what has been added and bracketing what has been deleted. Additionally, all amendments have been shaded.

In the Specification

The paragraph at page 2, lines 1-2 has been amended as follows:

Table 1. Species and donor cell type used to produce cloned mammals.

Species	Cell type used to produce a nuclear transfer offspring (clones)		
	Embryonic	Fetal	Adult
Mouse	Cheong et al., [1994] 1993	None reported	Wakayama et al., 1998
Rabbit	Stice and Robl, 1988	None reported	None reported
Cattle	Prather et al., 1987	Cibelli et al., 1998	Kato et al., 1998
Sheep	Willadsen, 1986	Campbell et al., 1996	Wilmut et al., 1997
Pig	Prather et al., 1989	Onishi et al., 2000	Polejaeva et al., 2000

The paragraph at page 2, lines 3-8 has been amended as follows:

Citations: Campbell et al., *Nature*, 380, 64 (1996); Cheong et al., *Biol. Reprod.*, 48, 958 (1993); Cibelli et al., *Science*, 280, 1256 (1998); Kato et al., *Science*, 282, 2095 (1998); Onishi et al., *Science*, 289, 1188 (2000); Prather et al., *Biol. Reprod.*, 37, 859 (1987); Polejaeva et al., *Nature*, 407, 86 (2000); Prather et al., *Biol. Reprod..*, 41, 414 (1989); Stice et al., *Biol. Reprod.*, 39, 657 (1988); Wakayama et al., *Nature*, 394, 369 (1998); Willadsen [et al.], *Nature*, 320, 63 (1986); and Wilmut et al., *Nature*, 385, 810 (1997).

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The paragraph at page 3, lines 14-19 has been amended as follows:

Improvements in oocyte activation in various species have been vigorously pursued (reviewed in Prather et al., *Theriogen.*, 51, 498 (1999)). Progress has been made by increasing calcium and/or decreasing protein phosphorylation in the oocyte (mice, [Szöllosi]Szöllösi et al., *J. Cell Sci.*, 104, 861 ([1994]1993); cattle, Susko-Parrish et al., *Dev. Biol.*, 166, 729 (1994) and Susko-Parrish et al., (U.S. Patent 5,496,720)).

The paragraph at page 15, line 24 to page 16, line 9 has been amended as follows:

Enucleation

Oocytes may be enucleated before introduction of donor genetic material. Enucleation of oocytes may be accomplished microsurgically using a micropipette to remove the polar body and the adjacent cytoplasm, or by chemical treatment (see, for instance, Baguisi et al., *Theriol.*, 53, [290]209 (2000). If enucleation is performed prior to introduction of donor genetic material, it may be conducted using methods previously described for enucleating MII oocytes (Tao et al., *Anim. Reprod. Sci.*, 56, 133-41 (1999)) or by methods such as described by Goto et al., (*Anim. Sci. J.*, 70, 243-245 (1999)). The oocytes may then be screened to identify those successfully enucleated. This screening can be done by staining the oocytes with a detectable marker that specifically binds to DNA (for instance, 1 μg/ml 33342 Hoechst dye in HEPES buffered hamster embryo culture medium (HECM, [Seshagine]Seshagiri et al., *Biol. Reprod.*, 40, [544]599-606, (1989)), and then viewing under ultraviolet irradiation for less than 10 seconds either the oocytes or the cytoplasm and maternal genetic material removed during the enucleation procedure. The oocytes that have been successfully enucleated can then be placed in a suitable culture medium, e.g., TCM-199, G1/G2, or CR1aa plus 10% serum (Stice et al., U.S. Patent 5,945,577).

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The paragraph at page 16 line 15 to page 17, line 3, has been amended as follows:

If the oocyte used to produce the NT embryo was not enucleated, then the NT embryo can be enucleated. Within the NT embryo, the maternal genetic material can be distinguished from the donor genetic material by, for instance, the position of the donor nucleus within the NT embryo, formation of the first polar body, or a combination thereof. The known location of the donor genetic material within the NT embryo is based on where it was placed in the perivitelline space in relation to the location of the maternal genetic material. The maternal genetic material is near the opening placed in the zona pellucida during transfer of the donor genetic material, preferably the donor genetic material is placed away from that area. Therefore that area of cytoplasm (near the opening in the zona) can be removed via either enucleation pipette or by expulsion of cytoplasm through the opening in the zona, preferably by enucleation pipette (see, e.g., Prather et al., *Biol. Reprod.*, 37, 859 (1987); and Goto et al., *Anim. Sci. J.*, [20]<u>70</u>, 243-245 (1999)). With regard to the second method, in some cases the oocyte may progress in meiosis to MII after introduction of the donor genetic material. If so, then the first polar body can also be used as landmark to find the maternal genetic material. Hoechst dye can be used to visualize genetic material, including confirming the presence of the maternal genetic material in the removed cytoplasm. These methods may be used alone or in conjunction with each other to verify location of chromosomes and verify enucleation of the oocyte.

The paragraph at page 18, lines 14-31 has been amended as follows:

Bovine oocytes and NT embryos may be activated by the method of Yang et al. (*Biol. Reprod.*, [42]<u>46</u>(Suppl 1), 117 (1992)), more preferably, by exposing bovine oocytes to about 1 μM to about 100 μM ionomycin, preferably about 50 μM ionomycin, for 10 minutes and about 1 μg/ml to about 100 μg/ml cycloheximide, preferably about 10 μg/ml cycloheximide, for about 2 hours to about 10 hours, preferably about 6 hours. Preferably, bovine oocytes and NT embryos

are activated by exposure to agents that increase the level of cations in the cell, followed by exposure to agents that agents that decrease protein synthesis in the cell and/or agents that are microfilament inhibitors. Most preferably, bovine oocytes and NT embryos are exposed to about 1 μM to about 100 μM calcium ionophore, preferably about 5 μM calcium ionophore, for about 10 minutes. This is followed by incubation in about 1 μg/ml to about 10 μg/ml cytochalasin B, preferably about 5 μg/ml cytochalasin B, and about 1 μg/ml to about 100 μg/ml cycloheximide, preferably about 10 μg/ml cycloheximide, for about 1 hour. This is followed by incubation in about 1 μg/ml to about 100 μg/ml cycloheximide, preferably about 10 μg/ml cycloheximide, for about 5 hours. Preferably, after the activation treatments, bovine NT embryos are cultured in BARC medium (Powell et al., *Theriogen.*, 55, 287 (2001)).

The paragraph at page 19, line 31 to page 20, line 16 has been amended as follows:

An activated NT embryo may be transferred immediately into a recipient animal or cultured for up to about 8 days in, for instance, KSOM medium, NCSU-23 medium, BARC medium, G1.2/G2.2 culture medium, or others well known to the art (see for instance Stice et al., U.S. Patent 5,945,577; Wells et al., *Biol. Reprod.*, 60, 996-1005 (1999); and Tao et al., *Anim. Reprod. Sci.*, 56, 133-41 (1999)). Preferably, an activated NT embryo is cultured for between about 12 hours to about 36 hours (for porcine NT embryos) or for about 7 to about 8 days (for bovine NT embryos). Then, intact NT embryos (some cleaved) are transferred into a synchronous recipient animal, i.e., the transferred NT embryo is at the same stage, or about a day before or a day after, as a fertilized embryo would be in the recipient. For pigs, from about one to about 300 NT embryos can be transferred into each recipient female but typically about 50 to about 150 embryos are transferred and ideally 100 embryos are transferred. Methods of surgical and non-surgical transfer in animals is well known in the art. For instance, surgical and non-surgical transfer in pigs is described by Curnock et al.,(*Amer. J. Vet. Res.*, 37, 97-98 (1976)),

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and Hazeleger et al., (*Theriogenol.*, 51, 81-[91]90 (1999)). Preferably, the animal is of the same species as the donor genetic material of the NT embryo.

The paragraph at page 30, lines 4-17 has been amended as follows:

Fusion was performed in a 100 mm dish filled with 15 ml of Zimmermann fusion medium (Zimmermann et al., *J. Membrane Biol.*, 67, 165-182 (1982)). Two stainless steel wires (100 μm diameter) attached to micromanipulators were used as electrodes. The single cell-oocyte complex was sandwiched between the electrodes and oriented with the contact surface between the enucleated oocyte and the donor cell perpendicular to the electrodes. The distance between the electrodes was about 100 μm. Membrane fusion was induced by applying a single direct current pulse of 250 V/mm for a duration of 20 μseconds with a prepulse of alternating current field of 5 V, 1 MHz for 2 seconds using an LF 101 Fusion Machine (TR Tech Co., Tokyo, Japan). Following the fusion, the NT embryos were washed in G1.2 medium (Vitrolife, Inc., Englewood Colorado) (Gandhi et al., *Mol. Reprod. Dev.*, 58, 269-275 (2001)) and cultured for a period of 1 hour in 100 μl of the same medium. Fusion was then determined by microscopic examination.

The paragraph at page 34, line 30 to page 35, line 10 has been amended as follows:

When transferred into recipient animals, the NT embryos are cultured for between about 12 hours to about 36 hours and then intact NT embryos (some cleaved) are transferred into a synchronous recipient gilt or sow. One to 300 NT embryos can be transferred into each recipient female but typically about 50 to about 150 embryos are transferred and ideally about 100 embryos are transferred. Methods of surgical and non-surgical transfer in pigs are well known in the art (Hazeleger et al., (*Theriogenol.*, 51, 81-[91]90 (1999)). Ultrasound and non-return to estrus are used to determine which recipients are pregnant. NT fetuses if needed for tissue or

cell transplantation can be harvested during the pregnancy through surgical recovery. If live pigs are desired the pregnancy last approximately 114 days and some pigs may require neonatal assistance in the form or oxygen supplementation and other interventions (Hill et al., *Theriogenol.*, 51, 1451 (1999)).

The paragraph at page 37, line 23 to page 38, line 13 has been amended as follows:

Oocyte preparation

In vitro maturation of bovine immature oocytes and enucleation were performed as described previously (Cibelli et al., *Science*, 280, 1256 (1998); Wells et al., *Biol. Reprod.*, 60, 996-1005 (1999); and Kubota et al., *Proc. Natl. Acad. Sci. USA*, [90]97, 990-995 (2000)). Briefly, bovine cumulus-oocyte complexes (COCs) were recovered by aspiration of small antral follicles on ovaries obtained from a slaughterhouse. Only COCs with a compact, nonatretic cumulus oophorus-corona radiata and a homogenous ooplasm were selected. They were matured in TCM 199 (Gibco Inc, Grand Island, New York) supplemented with 10% FBS, 50 μg/ml sodium pyruvate, 1% (volume:volume) penicillin/streptomycin (10,000 Units/ml penicillin G, 10,000 μg/ml streptomycin), 1 ng/ml rIGF-1 (Sigma), 0.01 Units/ml bLH and 0.01 Units/ml bFSH (Sioux Biochem. Sioux Center, Iowa) in four-well plates overlaid with mineral oil. Maturation was performed at 39°C in a humidified 5% CO_2 in air for 16-18 hours. After maturation, the cumulus-corona was totally removed by vortexing COCs in TL HEPES medium containing 100 Units/ml hyaluronidase (Sigma). Oocytes maturated for 16-18 hours were enucleated in MII phase with a 15 μm (internal diameter) glass pipette (Ependorf Munich, Germany) by aspirating the first polar body and MII plate in a small volume of surrounding cytoplasm in TL HEPES supplemented with 7.5 μg/ml Cytochalasin B (Sigma). The oocytes were previously stained in TL HEPES containing 2 μg/ml Hoechst 33342 and 7.5 μg/ml Cytochalasin B for 10-15 minutes. Enucleation was performed under ultraviolet light to ensure removal of oocyte chromatin.

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The paragraph at page 38, lines 15-26 has been amended as follows:

Fusion, activation, and culture of NT units

Oocyte-granulosa NT units were fused by using a needle-type electrode (Miyoshi et al., *Biol. Reprod.*, 62, 1640-1646 (2000); Goto et al., *Anim. Sci. J.*, 70, 243-245 (1999)) in Zimmermann's fusion medium (Zimmermann et al., *J. Membrane Biol.*, 67, 165-182 (1982)). The single cell-oocyte NT unit was sandwiched between two wires arranged in a straight line and attached to micromanipulators. The contact surface between the oocyte and the donor cell was parallel to the electrodes. The distance between the electrodes was approximately 150 μm (the diameter of the oocytes). A single direct current pulse of 40 V for a duration of 20 μseconds was applied. Following the pulse, the complexes were cultured in TCM 199 supplemented with 10 % FBS for 2 hours and fusion rates were determined.

The paragraph at page 38, line 27 to page 39, line 6 has been amended as follows:

Activation of NT embryos was performed as described previously (Goto et al., *Anim. Sci. J.*, 70, 243-245 (1999)[; Lui et al., *Mol. Reprod. Dev*, 56, 145-148 (1998)]) after modification. Briefly, 2 hours after fusion, NT embryos were exposed to 5 μM calcium ionophore (free acid, Sigma) for 10 minutes, followed by incubation in TCM 199 supplemented with 10% FBS, 5 μg/ml Cytochalasin B (Sigma), and 10 μg/ml Cycloheximide (Sigma) for 1 hour at 39°C in 5 % CO_2 in air and in TCM 199 supplemented with 10 % FBS and 10 μg/ml Cycloheximide for 5 hrs at 39°C in 5 % CO_2, 5 % O_2 and 90 % N_2. After the activation treatments, NT embryos were cultured in BARC medium (Powell et al., *Theriogen.*, 55, 287 (2001)) in four-well plates overlaid with mineral oil at 39°C in 5 % CO_2, 5 % O_2 and 90 % N_2 for 7-8 days.